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Adenovirus Early Region 4 Encodes Functions Required for Efficient DNA Replication, Late Gene Expression, and Host Cell Shutoff

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To delineate the function of adenovirus early region 4 (E4) gene products, we constructed a set of mutant viruses which carry defined lesions within this coding region. Deletion and insertion mutations within six of seven known E4 coding regions had no measurable effect on virus growth in cultured cells. A variant carrying a deletion within the last coding region (encoding a 34,000-molecular-weight polypeptide) was modestly defective, and a mutant lacking the majority of the E4 region was severely defective for growth. The phenotypes of the two defective mutants are similar and complex. Both display perturbations in DNA replication, translation of the E2A mRNA, accumulation of late viral mRNAs, and host cell shutoff.

The adenovirus type 5 (Ad5) early region 4 (E4) transcription unit lies between about 91.3 and 99.1 map units on the viral chromosome and is transcribed in the leftward direction (see Fig. 2). The nucleotide sequence of the closely related adenovirus type 2 reveals seven open translational reading frames of which five contain their own AUG start codons (12, 15). A family of differentially spliced mRNAs originates from this region (4, 9, 28), and E4-specific cDNA clones have been isolated and characterized (11, 29) which encode all but one of the seven open reading frames (see Fig. 2). Three E4 polypeptides have been identified within infected cells and mapped to specific coding regions (10, 22, 23). In vitro translation of E4-selected mRNAs has assigned a number of additional polypeptides to this region (20).

Challberg and Ketner (6) have isolated and propagated a defective E4 mutant (H2*dl*807, which lacks 82.5 to 95 map units) by using a helper virus. Although interpretation of the *dl*807 phenotype was complicated by the fact that its deletion extends outside of E4, mutant-infected cells exhibited abnormal synthesis of late viral polypeptides.

To further delineate the role of region E4 gene products, we constructed a set of Ad5 mutants which carry defined lesions within this coding region. Deletions and insertion mutations within six of seven known E4 coding regions had no measurable effect on virus growth in cultured cells. A variant carrying a deletion within the last coding region (H5*dl*355, which lacks 14 base pairs within the segment encoding a 34,000-molecular-weight polypeptide [34K polypeptide]) was modestly defective for growth, and a mutant lacking the majority of the E4 region (H5*dl*366) was severely defective. *dl*366 could be propagated only on W162 cells, a monkey kidney (Vero) cell line produced by Weinberg and Ketner (30) which contains and expresses E4.

The phenotypes of the two defective mutants are similar. Viral DNA replication is delayed in *dl*355-infected cells and reduced in *dl*366-infected cells. Although E2A-specific mRNA accumulates to normal levels, the E2A-specific polypeptide is substantially overproduced in mutant-infected cells. Some late viral mRNAs accumulate to reduced levels, and the shutoff of host cell metabolism occurs more slowly than in wild-type virus-infected cells.

MATERIALS AND METHODS

Plasmids, viruses, and cells. The recombinant plasmid pXbaC contains the Ad5 restriction endonuclease *Xba*I C fragment (84.3 to 100 map units) inserted between the unique *Hin*dIII and *Pvu*II sites of pBR322 (see Fig. 1). The *Hin*dIII site is lost and fused to the right end of the Ad5 segment, while the *Pvu*II site is lost and fused to an intact *Xba*I cleavage site at 84.3 map units on the Ad5 chromosome.

H5*wt*300 is a plaque-purified derivative of a virus stock originally received from H. Ginsberg. The mutant viruses produced in this study and the coordinates of their lesions (determined by nucleotide sequence analysis) are displayed in Fig. 2. In addition to their E4-specific alterations, these viruses all lack the 78.5- to 84.3-map-unit *Xba*I fragment which lies within early region 3. Thimmappaya et al. have previously shown that viruses carrying this deletion are phenotypically wild type (*dl*324; reference 27). Two independent isolates of *dl*355 were generated and found to have identical phenotypes. Virions prepared by equilibrium density centrifugation (17) were used for all infections. Virion concentration was measured by disruption of virus particles (0.1% sodium dodecyl sulfate) and determination of A_{260} (one absorbance unit = 10^{12} particles per ml). All infections were done at 200 particles per cell (about 10 PFU per cell) unless otherwise noted.

The 293 cell line (a human embryonic kidney cell line transformed with a DNA fragment carrying the left 11% of the Ad5 genome [13]) and human A549 cells were both obtained from H. Young and maintained in medium containing 10% calf serum. The W162 cell line (a Vero line carrying an integrated copy of the Ad5 E4 region [30]) and the parental Vero cell line were obtained from G. Ketner and propagated in medium containing 10% calf serum. Spinner culture HeLa cells and a second HeLa line which grows in monolayer culture were obtained from the American Type Culture Collection and grown in medium containing 7.5 and 10% calf serum, respectively.

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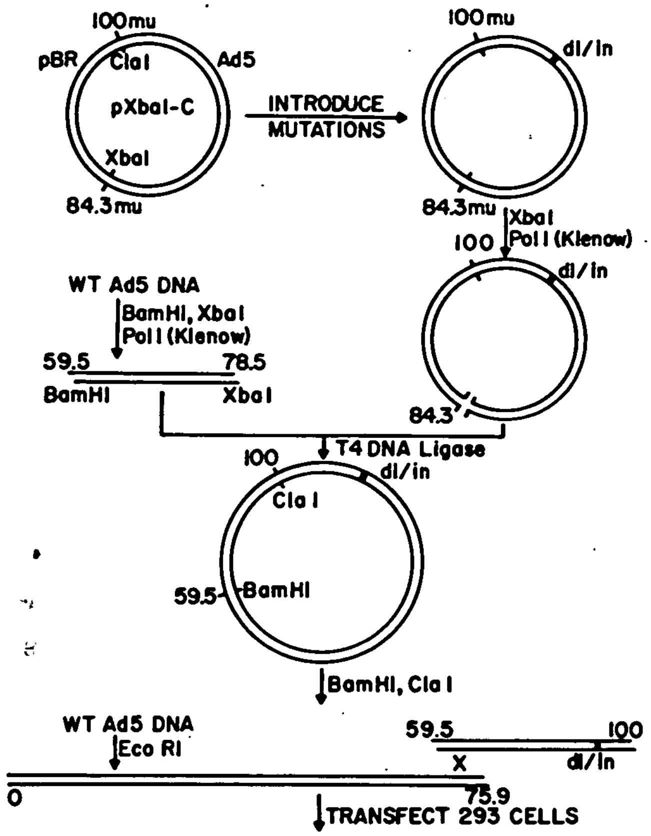

FIG. 1. Flow diagram depicting the series of manipulations employed to generate Ad5 variants carrying mutations within region E4.

DNA replication rates. Viral and cellular DNA replication rates were assayed by labeling HeLa Spinner cells at a concentration of 5×10^6 cells per ml for 1 h with [^{3}H]thymidine (100 μCi/ml; 50 Ci/mmol) at various times after infection. Total DNA was extracted and analyzed either by electrophoresis after digestion with a restriction endonuclease or by equilibrium density centrifugation (27).

RNA preparation and analysis. The protocols for cytoplasmic poly(A)-containing RNA isolation from infected HeLa cells and Northern-type analysis have been described previously (14). Recombinant plasmids carrying region-specific Ad5 DNA segments were used as probe DNAs (in map units): E1B, 5.6 to 7.9; E2A, 59.5 to 75.9; L1, 31.5 to 37.3; L2, 41 to 50.1; L3, 52.5 to 58.5; L4, 70.7 to 75.9; L5, 84.3 to 91.9.

For measurement of transcription rates, infected HeLa Spinner cells were pulse-labeled at a concentration of 5×10^6 cells per ml for 10 min with [^{3}H]uridine (200 μCi/ml; 50 Ci/mmol). Nuclear RNA was prepared by the method of Chirgwin et al. (8) and then subjected to LiCl precipitation. RNAs were hybridized to single-stranded probe DNAs bound to nitrocellulose filters (100 μg of genome equivalents per filter) essentially as described by Nevins (21). After the first hybridization, a second filter was added to ensure quantitative results. Recombinant M13 viral DNAs carrying region-specific Ad5 DNA segments were used as probes (in map units): E2A, 59.5 to 70; L1 through L4, as above for Northern probes; L5, 88 to 91.9.

Analysis of polypeptides. Cultures were labeled in medium lacking methionine supplemented with 2% calf serum and [^{35}S]methionine (50 μCi/ml; 1,100Ci/mmol) for 1 h unless otherwise noted. Preparation of cellular extracts, immunoprecipitations, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out as described by Sarnow et al. (24). The E1B-55K-specific monoclonal antibody (2A6) and the Ad2 tumor serum (Ad2) utilized were gifts of A. Levine and have been described previously (25).

RESULTS

Construction and propagation of mutants. All mutations were initially constructed in a recombinant plasmid containing the *Xba*I C fragment (84.3 to 100 map units) of the Ad5 chromosome (Fig. 1). With the exception of *dl*366, all mutations were introduced by restriction endonuclease cleavage of the parent plasmid, followed by *Bal* 31 exonuclease digestion or repair with DNA polymerase (Klenow fragment) and then religation to create deletions or insertions, respectively. *dl*366 was constructed by excision of the *Sma*I H fragment (91.9 to 98.3 map units). To facilitate reconstruction back into the viral chromosome, the viral

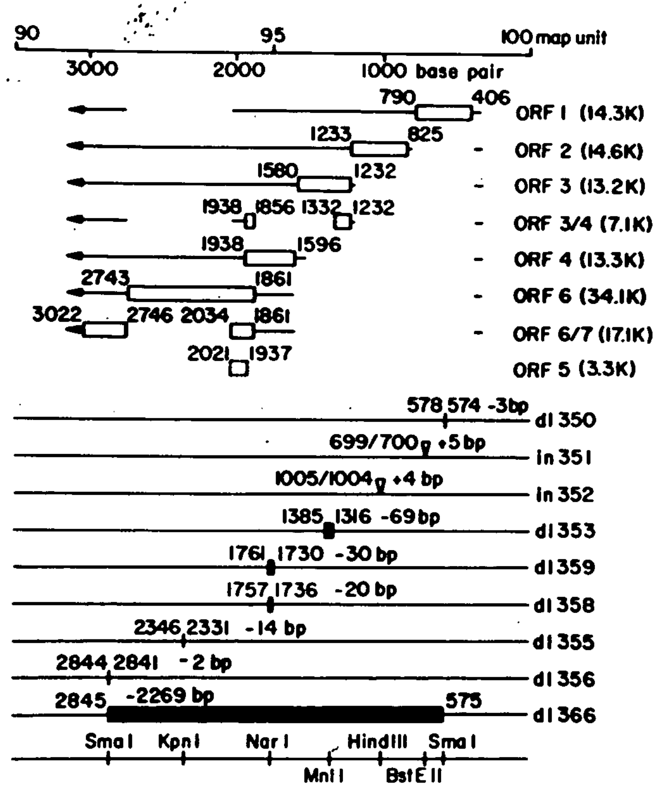

FIG. 2. Physical map of the E4 mRNAs, coding regions, and mutations. The top of the figure positions the map in terms of map units and nucleotide sequence relative to the right end of the viral chromosome (12). mRNAs which have been identified by cDNA cloning (11, 29) are indicated by lines, introns are indicated by spaces, and polypeptide coding regions are indicated by open rectangles. ORF designates open reading frame. The open rectangle outlined with a broken line identifies an ORF which contains no AUG and for which no mRNA has been described. Deletion mutations are represented by solid rectangles, and insertion mutations are represented by triangles. The first and last nucleotides of ORFs as well as the nucleotides which are present bracketing mutational alterations are designated by nucleotide sequence numbers. bp, Base pairs.

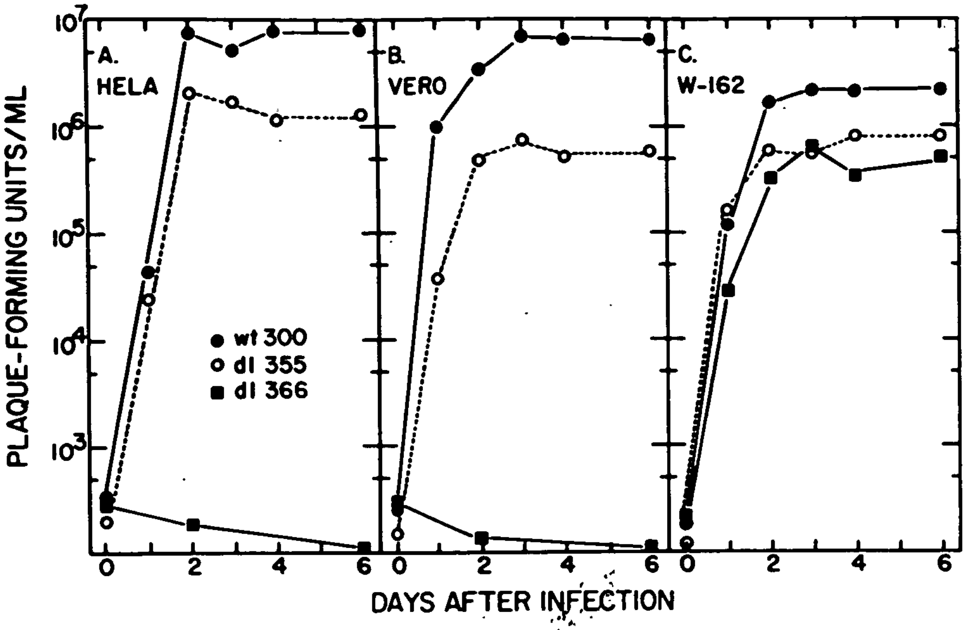

FIG. 3. Growth kinetics of mutant and wild-type viruses in HeLa, Vero, and W162 cells. Cells were infected at a multiplicity of 200 particles per cell, and the virus yield was assayed at the times indicated by plaque assay on W162 cells. Symbols: ●, *wt*300; ○, *dl*355; ■, *dl*366.

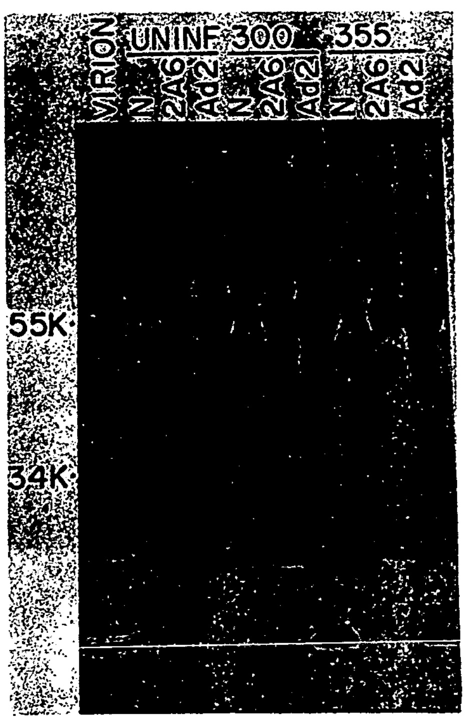

FIG. 4. Analysis of the E4-34K polypeptide produced in *dl*355- and *wt*300-infected 293 cells. Cells were labeled for 1 h with [^{35}S]methionine at 8 h after infection. Extracts were prepared, and immunoprecipitations were carried out with an E1B-55K-specific monoclonal antibody (2A6), a polyclonal hamster tumor serum (Ad2), or a normal hamster serum (N). Electrophoresis was in an 18% polyacrylamide gel containing sodium dodecyl sulfate. Bands representing the E1B-55K and E4-34K moieties are indicated beside the autoradiogram. UNINF, Uninfected cells; 300, *wt*300-infected cells; and 355, *dl*355-infected cells.

DNA segment extending from 59.5 to 78.5 map units was inserted into each mutant clone (Fig. 1). Mutant viruses were then generated by overlap recombination (7, 16). In this procedure, two overlapping DNAs are used to transfect cells, neither of which represents an intact viral genome. Recombination occurs within the transfected cells in the overlap region to generate an intact and infectious viral chromosome. In this case, a 0- to 75.9-map-unit segment was derived from *wt*300 DNA, and a 59.5- to 100-map-unit segment was provided by the plasmid DNA. Recombination between 59.5 to 75.9 map units fixed the E4-specific mutations into the viral chromosome.

All mutant viruses were propagated on the 293 cell line except *dl*366, which was grown on the W162 cell line. Deletions in the E4 region were precisely positioned by DNA sequence analysis, and the coordinates of each mutation are shown in Fig. 2.

The only mutant viruses which exhibited a reduced yield after infection of HeLa cells were *dl*355 and *dl*366 (Fig. 3). Apparently, the preponderance of E4-specific polypeptides were not essential for efficient viral multiplication in cultured cells. The growth kinetics and final yield of *dl*355 were reduced as compared with those of *wt*300, while *dl*366 failed to produce detectable progeny. *dl*366 was propagated on W162 cells, which contain and express region E4 (30). Although these cells complemented the severe *dl*366 growth defect, neither mutant virus grew quite as well as *wt*300. Further, both *dl*355 and *dl*366 produced smaller plaques than wild-type virus on these cells. Some component of their defects is not entirely complemented by the resident viral DNA sequences in W162 cells.

The final yield in wild-type virus-infected HeLa cells was about 10^7 PFU per ml when titers were obtained by plaque assay on W162 cells (Fig. 3). This was 100-fold lower than that expected for wild-type virus. To account for this discrepancy, the wild-type virus yields were assayed by plaque formation on 293 cells. The final yield determined on the 293 cell line was in the range of 10^9 PFU per ml (data not shown).

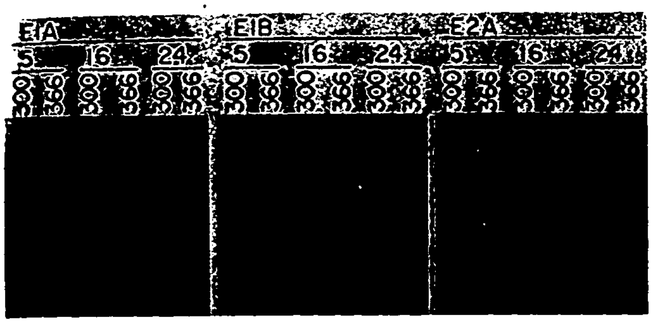

FIG. 5. Northern-type analysis of early viral mRNA species present in HeLa cells infected with *dl*366 (366) or *wt*300 (300) viruses. Cytoplasmic polyadenylated RNA was prepared at 5, 16, and 24 h after infection. Equal quantities of these RNAs were subjected to electrophoresis, transferred to nitrocellulose, and hybridized to region-specific probe DNAs. The E2A mRNA was the only species observed at 5 h after infection. The probe DNA (59.5 to 75.9 map units) overlaps the L3 and L4 coding regions whose products are evident in the 16- and 24-h samples.

Thus, the low yields seen in this experiment are a reflection of the reduced plaquing efficiency of Ad5 on the W162 cell line compared with that on the 293 cell line.

dl355 does not encode the E4-34K polypeptide. Whereas *dl*366 should fail to code any E4-specific polypeptides, the lesion in *dl*355 should specifically affect synthesis of the E4-34K polypeptide (Fig. 2). This polypeptide exists in a complex with the E1B-55K polypeptide and can be identified by its coimmunoprecipitation with the E1B species (23). Accordingly, the 293 cell line was infected with mutant and wild-type viruses and labeled with [^{35}S]methionine, and cell lysates were subjected to immunoprecipitation with monoclonal or polyclonal antiserum specific for the E1B-55K polypeptide. Electrophoretic analysis of the immunoprecipitates showed that the E4-34K polypeptide was present in *wt*300-infected cells but not in *dl*355-infected cells (Fig. 4). A truncated 188-amino-acid polypeptide could be synthesized by *dl*355. This fragment was not detected in infected cells in this experiment or when HeLa cells were infected at high multiplicities of infection with short labeling periods (15 min) (data not shown). One of two possibilities could explain this result. Either the truncated version of the polypeptide is degraded too rapidly for detection or it does not associate with the E1B-55K polypeptide in a protein complex.

The dl355 and dl366 phenotypes are complex. To elucidate the physiological consequences of the E4 mutants, we compared their replication cycles with those of the wild-type parent, *wt*300, in HeLa cells.

To assay expression of early genes, cytoplasmic poly(A)-containing RNA was prepared from HeLa cells infected with mutant or wild-type viruses and subjected to Northern-type analysis. There was no difference at 5 h after infection with either *dl*355 or *dl*366 in levels or variety of those early mRNAs tested (results for *dl*366 are shown in Fig. 5). Near normal levels of *dl*355-coded early mRNA species were observed at later times after infection, but somewhat less early mRNA accumulated in *dl*366-infected cells than in *wt*300-infected cells at 16 and 24 h after infection (Fig. 5). This reduction in early mRNAs likely results from reduced template copy number in *dl*366-infected cells (discussed below).

Viral DNA replication was measured by briefly labeling infected HeLa cells at 16 and 24 h after infection, isolating total high-molecular-weight DNA, digesting the DNA with the *Hin*dIII endonuclease, and separating the resulting fragments by electrophoresis (Fig. 6). Although the rate of *dl*355 synthesis appeared similar to that of *wt*300, *dl*366 replication was reduced about 10-fold. In contrast to the wild-type DNA, mutant DNA preparations generated a smear of radioactively labeled cellular DNA fragments upon *Hin*dIII

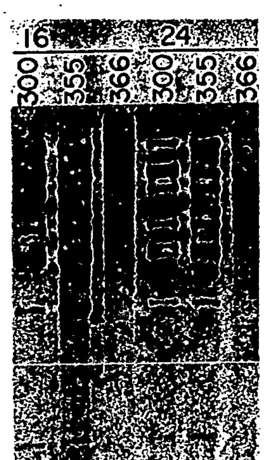

FIG. 6. DNA replication rates determined by electrophoretic analysis of labeled DNAs prepared from mutant (355 and 366) or wild-type (300) virus-infected HeLa cells. Infected cells were labeled for 1 h with [^{3}H]thymidine at either 16 or 24 h after infection. Total high-molecular-weight DNA was prepared, cleaved with *Hin*dIII, and subjected to electrophoresis. The radioactive DNA bands were visualized by fluorography.

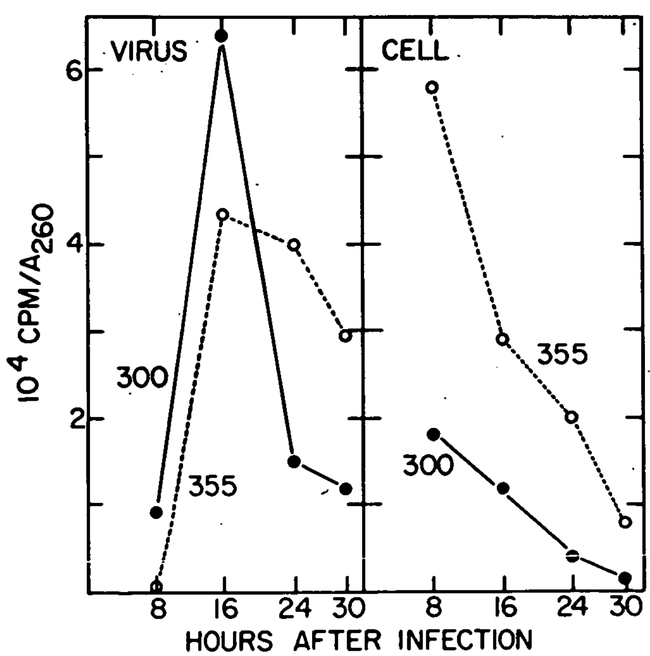

FIG. 7. DNA replication rates determined by equilibrium density centrifugation of labeled DNAs prepared from *dl*355- or *wt*300-infected HeLa cells. Infected cells were labeled with [^{3}H]thymidine for 1 h at the indicated times after infection, high-molecular-weight DNA was prepared, DNAs of viral and host cell origin were separated by equilibrium density centrifugation, the total radioactivity in each species was determined, and the results were plotted as a function of time. Symbols: ●, *wt*300; ○, *dl*355.

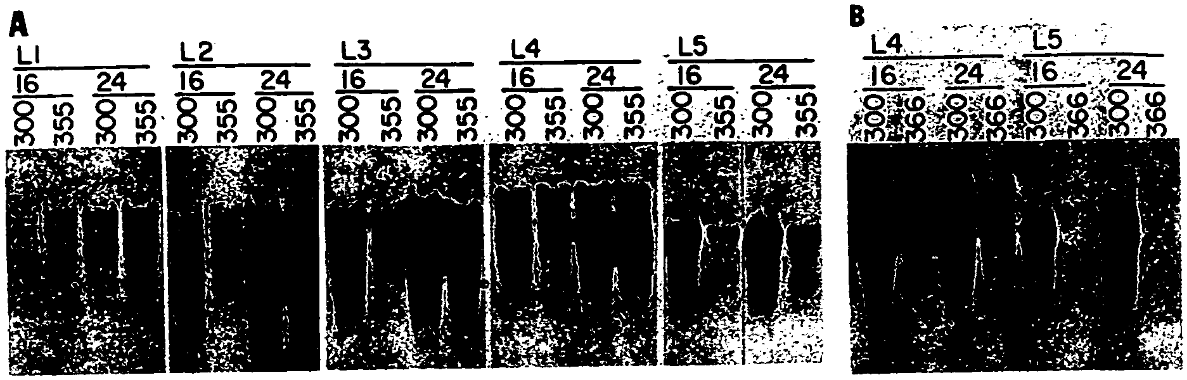

FIG. 8. Northern-type analysis of late viral mRNAs present in infected HeLa cells. Cytoplasmic polyadenylated RNAs were prepared at 16 and 24 h after infection with *wt*300 (300, A and B), *dl*355 (355, A), or *dl*366 (366, B) and processed as described in the legend to Fig. 5. The fastest-migrating band detected with the L5-specific probe DNA (84.3 to 91.9 map units) was a truncated E3 mRNA; the more slowly migrating species was a doublet (seen in light exposures) representing L5 mRNAs.

cleavage. Evidently, cellular DNA replication in mutant-infected cells was not shut off as efficiently as in wild-type virus-infected cells. The replication defect seen for *dl*366 led us to examine viral DNA replication of the mutant *dl*355 by a more sensitive assay. At 8, 16, 24, and 30 h after infection with *dl*355 or *wt*300, cells were labeled with [^{3}H]thymidine for 1 h, total DNA was extracted, and viral DNA was separated from cellular DNA by equilibrium density centrifugation (Fig. 7). This assay revealed a modest delay in both the onset of viral DNA synthesis and the shutoff of host cell replication. These delays were reproduced in three separate experiments. We conclude that E4-specific products are required for optimal rates of viral DNA replication.

Next, transcription rates for the E2A and L1 to L5 coding regions were measured late (16 h) after infection of HeLa cells. Cells were pulse-labeled (10 min) with [^{3}H]uridine, and nuclear RNA was prepared and hybridized to an excess of single-stranded probe DNA. The data from the hybridization are presented in Table 1. Transcription rates for late coding regions in *dl*355-infected cells were 35 to 45% of those seen in wild-type-infected cells, and rates in *dl*366-infected cells were reduced to only 9 to 10% of those in wild-type-infected cells. Transcription rates for the early unit (E2A) were also reduced but to a lesser extent. Transcriptional activity parallels the reductions in replication of mutant DNAs at this time (Fig. 6 and 7). Thus, it seems likely that the reduction in transcription rates reflects a decrease in template copy number.

TABLE 1. Transcription rates at 16 h after infection[a]

Probe	cpm/map unit in cells infected with:			Ratio	
	*wt*300	*dl*355	*dl*366	*dl*355/*wt*300	*dl*366/*wt*300
L1	5,434	2,458	543	0.452	0.100
L2	2,482	965	249	0.389	0.104
L3	3,689	1,408	357	0.382	0.097
L4	2,709	940	241	0.347	0.089
L5	2,316	848	220	0.366	0.095
E2A	682	458	293	0.672	0.430

[a] HeLa cells were pulse-labeled (10 min) with [^{3}H]uridine at 16 h after infection. Nuclear RNA was prepared and hybridized to region-specific, single-stranded probe DNAs immobilized on nitrocellulose filters. To account for differences in the size of probe DNAs, results have been normalized to counts per minute per map unit (360 base pairs) of probe DNA.

Steady-state levels of late mRNAs were monitored by Northern-type analysis at 16 and 24 h after infection of HeLa cells. *dl*355-infected cells contained nearly normal levels of some late mRNAs (Fig. 8A, region L1) and moderately reduced levels of other regions (Fig. 8A; region L5 was reduced almost fivefold). As expected, more dramatic differences were observed in *dl*366-infected cells. Densitometric scanning of the autoradiogram displayed in Fig. 8B indicated that L4 and L5 mRNAs were reduced 2.5- and 23-fold, respectively, in *dl*366 as compared with *wt*300-infected cells. Thus, in mutant-infected cells, accumulation of mRNAs corresponding to some late coding regions (L3 and L5) was more severely affected than accumulation from other regions (L1, L2, and L4). Since transcription rates were uniformly reduced but steady-state levels of mRNAs were variable in mutant-infected cells, we conclude that E4 gene products function posttranscriptionally to influence accumulation of viral mRNAs.

Late viral polypeptide synthesis was examined by labeling infected HeLa cells with [^{35}S]methionine at 16 and 24 h after infection and subjecting cell lysates to polyacrylamide gel electrophoresis. For the most part, polypeptide patterns were predictable from the mRNA analyses (Fig. 9). Levels of most late polypeptides were modestly reduced in *dl*355-infected cells and more severely reduced in *dl*366-infected cells. However, in contrast to late polypeptides, the E2A-72K polypeptide was overproduced in cells infected with either mutant. The overproduction was dramatic in the case of *dl*366 at 24 h after infection, in which the E2A-72K polypeptide appeared to be the predominant protein moiety synthesized in the infected cell (Fig. 9). The identity of the polypeptide was confirmed by immunoprecipitation with a 72K-specific monoclonal antibody (data not shown). The reason for the overproduction is unclear, since the E2A mRNA accumulates to normal levels in mutant-infected cells (Fig. 5). The final point to be made from analysis of late polypeptide synthesis is that both mutants appeared inefficient in shutoff of host protein synthesis. This is most readily observed in *dl*366-infected cells at 24 h after infection (Fig. 9).

The host cell influences the *dl*355 phenotype. As discussed above, we could observe no phenotype for most of our E4 mutants in the 293 cell line. It seemed possible these mutants might display a phenotype in a different human cell line. Accordingly, late polypeptide synthesis was monitored in a

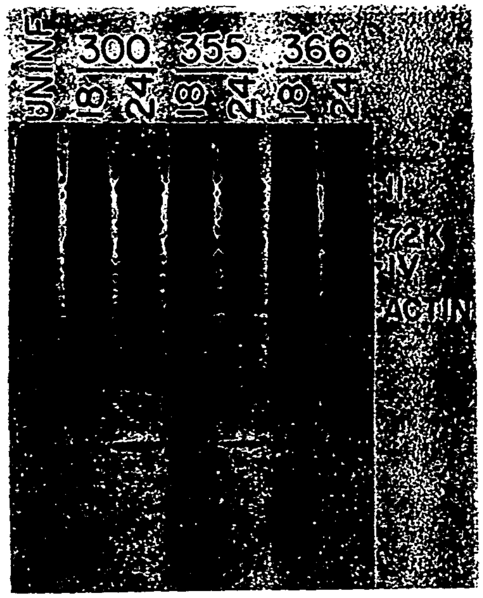

FIG. 9. Electrophoretic analysis of polypeptides synthesized in HeLa cells infected with mutant (355 and 366) and wild-type (300) viruses. At 18 and 24 h after infection, cultures were labeled for 1 h with [^{35}S]methionine. Cell extracts were prepared, and portions were subjected to electrophoresis in a 12.5% polyacrylamide gel containing sodium dodecyl sulfate. Bands corresponding to cellular actin and several viral polypeptides are designated. The viral polypeptides indicated are: II, L3-coded II (hexon); 72K, E2A-coded 72K; and IV, L5-coded IV (fiber). Uninfected cells (UNINF) are shown for comparison.

variety of human cells. Unfortunately, none of the apparently normal mutants displayed a defect on any cell line tested (data not shown). However, *dl*355 proved to be more severely defective when grown in a HeLa monolayer cell line obtained from the American Type Culture Collection (designated HeLa-A) than in 293 or HeLa Spinner cells (Fig. 10). Underproduction of fiber (IV) polypeptide, overproduction of the E2A-72K polypeptide, and failure to shut off the host cell were all more extreme in these cells than in other lines tested (such as the A549 cells shown in Fig. 10). Thus, the host cell can modulate the phenotype of *dl*355.

DISCUSSION

Only two of nine mutant viruses we constructed to carry lesions in the adenovirus E4 region are compromised in their ability to grow in human cells. This observation suggests that many of the E4-specific gene products are not required for growth of Ad5 in cultured cells. There is precedent for nonessential genes in region E3 which can be deleted without markedly affecting viral growth in the laboratory (e.g., see references 5 and 27). Presumably, these genes play important roles during virus propagation in its natural host (the human) but not during replication in rapidly growing tissue culture cells.

One might argue that our mutational analysis failed to identify polypeptides which perform essential E4 functions. For example, it is possible that in-frame deletions (*dl*350, *dl*351, and *dl*353 remove 36, 3, and 69 base pairs, respectively) could remove nonessential domains of an otherwise essential polypeptide. Alternatively, the multiple E4 coding regions may produce products with related functions. Thus, critical functions could be missed since when one polypeptide is disrupted another can substitute for it. Finally, the wild-type growth of certain E4 mutants could result from a selection for viruses capable of growth which occurred during the reconstruction process. With the exception of *dl*366, all E4 variants were selected for growth on the 293 cell line. As a result, they could conceivably carry second-site mutations which compensate for their E4-specific lesions. The best argument against all of these possibilities rests on *dl*366. This virus was reconstructed in W162 cells and, therefore, was presumably generated under nonselective conditions. *dl*366 lacks all E4 coding regions but does not appear to have altered any functions unrelated to those perturbed by *dl*355, whose lesion lies entirely within the E4-34K region. Thus, although there could be functional reiterations of the E4-34K polypeptide, there is no reason to suspect that E4 codes additional non-E4-34K-related functions which are essential for growth in cultured cells.

Why is the defect exhibited by *dl*366 more severe than that observed in *dl*355-infected cells? Possibly, *dl*355 generates a partially functional fragment comprising the N-terminal half of the E4-34K polypeptide. This fragment, which cannot be coded by *dl*366, could ameliorate the *dl*355 phenotype. Alternatively, the E4 region may encode a second polypeptide, not altered in *dl*355, which can partially substitute for the E4-34K species. The 17.1K polypeptide, which could be synthesized from the mRNA carrying the fused 6/7 open reading frame (Fig. 2), is an excellent candidate for this functionally related species. This polypeptide would carry an N-terminal region identical to that of the 34K species fused to a novel C-terminal domain, and its synthesis would not be altered by the *dl*355 mutation. Genetic experiments are in progress to differentiate these possibilities.

FIG. 10. Electrophoretic analysis of polypeptides synthesized in A549 or HeLa-A cells infected with *dl*355 (355) or *wt*300 (300). Infected cells were labeled for 1 h at 32 h after infection and processed as described in the legend to Fig. 9. Uninfected cells (UN) are shown for comparison.

The phenotypes of *dl*355 and *dl*366 are complex but clearly related. The first point in the viral growth cycle at which we observe a perturbation is the onset of DNA replication. In *dl*355-infected cells it is delayed (Fig. 7), while in *dl*366-infected cells replication is reduced about 10-fold (Fig. 6). Thus, the E4-34K and perhaps additional functionally related E4 polypeptides play a role in viral DNA synthesis. An E4 product is not required in purified cell-free replication systems (reviewed in references 18 and 26). However, the in vivo E4 requirement is not absolute but rather modulates efficiency. So, it is not surprising that cell-free systems would not require the factor. Perhaps their activity would be enhanced by its addition. It is also possible that the E4 role in DNA replication is indirect. For example, the E4 product could affect levels of E2B products known to be involved in replication.

Late transcription rates are uniformly reduced across the major late transcription unit for both mutants (Table 1), and this is very likely a reflection of reduced template copy number. If there was no alteration in late mRNA metabolism beyond reduced transcription rates, one would predict a uniform reduction in steady-state levels of late mRNAs. However, some late mRNAs (e.g., L5) accumulate to lower levels than do others (e.g., L4) in mutant-infected cells as compared with wild-type virus-infected cells (Fig. 8A and B). This indicates there is a second transcriptional perturbation which occurs after production of the primary transcript and before accumulation of mature cytoplasmic mRNAs. Thus, E4-coded products influence either an RNA processing step or transport of the mature mRNA from nucleus to cytoplasm or stability of the mRNA in the cytoplasm. As was the case for DNA replication, this effect could be indirect, and further biochemical studies will be required to better understand the E4 role in late transcription.

The E2A-72K polypeptide is dramatically overproduced late after infection with either *dl*355 or *dl*366 (Fig. 9 and 10). E2A mRNA levels are normal at these times (Fig. 5), so the increased level of the 72K moiety must result from either an enhanced rate of synthesis or an increase in the half-life of the polypeptide. It is possible that certain of the abnormalities observed in *dl*355- and *dl*366-infected cells are due to E2A-72K overproduction.

Besides exhibiting alterations in viral gene expression, *dl*355 and *dl*366 fail to efficiently shut off host cell metabolism both at the level of DNA (Fig. 6 and 7) and protein (Fig. 9 and 10) synthesis. Shutoff of host cell protein synthesis occurs at two levels. In the nucleus, transcription of cellular genes is not substantially altered late after infection, but newly synthesized RNAs fail to appear in the cytoplasm (e.g., references 1 and 3). In the cytoplasm, steady-state levels of preexisting cellular mRNAs are little changed during infection, but they are not actively translated. Since we observed a posttranscriptional perturbation of viral mRNA metabolism (discussed above) as well as failure to shut off host gene expression, it is tempting to speculate that an E4 gene product plays a pivotal role in cytoplasmic mRNA accumulation within infected cells. Perhaps this product functions both to facilitate viral and inhibit cellular mRNA accumulation.

Logan et al. (19), Pilder et al. (S. Pilder, J. Logan, and T. Shenk, submitted for publication), and Babiss and Ginsberg (2) have described mutants which carry lesions in the E1B-55K polypeptide and display lytic growth phenotypes similar to those delineated here for *dl*355 and *dl*366. The similarity makes sense because the E1B-55K and E4-34K polypeptides have been shown to exist in a physical complex (23). The similar phenotypes indicate that these two polypeptides probably do function as a complex.

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Appendix B
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Classification of Human Adenoviruses

At least four different criteria have been used to organize the 31 serotypes of human adenoviruses into affinity groups that share common properties.

Oncogenicity

Trentin's observations on the oncogenicity of human adenovirus 12 were rapidly confirmed and extended to certain other human adenoviral serotypes and to other rodent species (Huebner et al. 1962, 1963; Yabe et al. 1962; Girardi et al. 1964; Rabson et al. 1964; Larsson et al. 1965; Pereira et al. 1965; Trentin et al. 1968). In addition, it was shown that the oncogenic potential of adenoviruses is not limited to strains isolated from humans; adenoviruses of simian (Hull et al. 1965; Rapoza et al. 1967), bovine (Darbyshire 1966), canine (Sarma et al. 1967), and avian (Sarma et al. 1965; Anderson et al. 1969a,b,c) origin all were found to induce tumors when injected into newborn rodents of several species.

However, not all human adenoviruses are tumorigenic in rodents and the oncogenic potential of those that are varies. Only a few types (12, 18, and 31) are strongly oncogenic and able to induce tumors rapidly in the majority of inoculated animals. Most of the remaining potentially oncogenic serotypes (3, 7, 8, 14, 21, and 24) have a very limited capacity to induce tumors. Furthermore, in contrast to the undifferentiated sarcomas induced by adenovirus 12 which develop within 3 months

of inoculation, the malignant lymphomas induced by other adenoviral serotypes appear only after an extended latent period of 9 to 23 months.

The remaining serotypes (including types 1, 2, 4, 5, 6, 9, 10, and 11) thus far have not manifested any tumorigenic potential. Why different serotypes should induce tumors at different frequencies is not known. Nevertheless, their oncogenic potential in newborn hamsters has been used in a classification scheme to define three arbitrary subgroups A, B, and C as highly oncogenic, weakly oncogenic, and nononcogenic, respectively (Huebner et al. 1965) (see Table 8.1).

DNA Homology

The human adenoviruses have also been classified into subgroups according to the base composition of their DNAs (Piña and Green 1965) and the extent of homology as measured by DNA:DNA and DNA:RNA hybridization (Lacy and Green 1964, 1965, 1967; Green 1970; Garon et al. 1973; Bartok et al. 1974). Again, the viruses fall into three groups which, perhaps surprisingly, correspond fairly well with those defined on the basis of oncogenicity. The base composition of the DNAs of viruses belonging to subgroup A is 48–49% G+C, that of viruses of subgroup B is 49–52% G+C, and that of viruses of subgroup C is 55–60% G+C (Piña and Green 1965; Green 1970). This correlation between G+C content and oncogenicity is

Table 8.1 Subgroups of human adenoviruses

Subgroup	Member serotype	Oncogenicity[a]	DNA:DNA homology[b] (%)	Hemagglutination subgroup[c]
A	12, 18, 31	high	80–85	IV
B	3, 7, 11, 14, 16, 21	weak	70–95	I
C	1, 2, 5, 6, 4	none	85–95	III
D	17, 19, 20, 22–28	none	n.d.	II

[a]Highly oncogenic serotypes cause tumors in almost all infected newborn mice within 2 months; weakly oncogenic serotypes induce tumors in some infected animals in 4–18 months. Data from Huebner (1967).
[b]Data from Lacy and Green (1964, 1965, 1967); n.d. = not determined.
[c]Data from Rosen (1960).

REPLICATION OF
WILD-TYPE ADENOVIRUS

The infection cycle of a wild-type adenovirus is illustrated in the following diagram: